Persistent Organic Pollutants in Matched Breast Milk and Infant Faeces Samples

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Abstract

Assessing blood concentration of persistent organic pollutants (POPs) in infants is difficult due to the ethical and practical difficulties in obtaining sufficient quantities of blood. To determine whether measuring POPs in faeces might reflect blood concentration during infancy, we measured the concentrations of a range of POPs (i.e. polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides (OCPs)) in a pilot study using matched breast milk and infant faecal samples obtained from ten mother-child pairs. All infants were breast fed, with 8 of them also receiving solid food at the time of faecal sampling. In this small dataset faecal concentrations (range 0.01 - 41ng/g lipid) are strongly associated with milk concentrations (range 0.02 - 230 ng/g lipid). Associations with other factors generally could not be detected in this dataset, with the exception of a small effect of age or growth. Different sources (external or internal) of exposure appeared to directly influence faecal concentrations of different chemicals based on different inter-individual variability in the faeces-to-milk concentration ratio R_{fm}. Overall, the matrix of faeces as an external measure of internal exposure in infants looks promising for some chemicals and is worth assessing further in larger datasets.

Keywords: Infant, POPs, blood concentration, breast milk concentration, faeces concentration

1. Introduction

The link between exposure to some persistent organic pollutants (POPs) and endocrine disrupting/ carcinogenic effects seems to be clear from laboratory studies on a range of biota (Tanabe, 2002; Li et al., 2006; Alonso-Magdalena et al., 2011; Bergman et al., 2013), and there is increasing evidence that exposure in early life is an important determinant of long-term disease risk (Nagayama et al., 2007; Ochiai et al., 2014). However, more large-scale epidemiological studies are needed for understanding the exposure window that can elicit effects and the extent of the contribution of lipophilic pollutants to the increased risk of incommunicable diseases in humans (Nickerson, 2006; Jorissen, 2007). Infancy is a critical stage of development and is associated with unique exposure pathways that can increase exposure to lipophilic pollutants (Solomon and Weiss, 2002). Nevertheless, exposure assessment in early life is challenging and complicated by reluctance of parents and investigators to take blood samples from infants. In addition, the small volume sample that may be obtained poses technical challenges for measuring analytes.

To avoid sampling blood, physiologically based pharmacokinetic (PBPK) models have been used to generate individualized toxicokinetic profiles of lipophilic pollutants in infants. Such models usually contain many variables, such as: the initial value (e.g., the concentration in meconium or umbilical cord blood), ongoing exposure values (e.g., concentrations in mother's breast milk), intrinsic elimination values (estimated from assembled data from longitudinal monitoring data or estimated via measuring excretion rate) and infants' growth rate (Lorber and Phillips, 2002; Verner et al., 2013). Each of these variables is uncertain, and consequently the modelled exposure of the infant can also be associated with considerable uncertainty. In this context, finding a non-invasive matrix that has a direct relationship with the blood concentration and can be measured repeatedly throughout infancy would improve exposure assessment of lipophilic pollutants in infants.

Evidence in adults suggests that the faecal concentration of some lipophilic pollutants (polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs), polychlorinated biphenyls (PCBs) and hexachlorobenzene (HCB)) is directly related to the blood concentration. Schrey et al. (1998) reported that faecal excretion of most PCDD/Fs examined exceeded the daily intakes from food (Schrey et al., 1998). Moser and McLachlan also found that altering dietary exposure did not influence faecal excretion of PCDD/Fs, HCB, and PCBs (Moser and McLachlan, 2001), which means faecal concentration was not affected by the concentration of POPs in diet. Further, Rohde et al. found a correlation between levels in blood and levels in faeces for PCDD/Fs over a very

broad concentration range (Rohde et al., 1999). To-Figueras and his team pointed out a strong correlation between HCB levels in faeces and in serum (To-Figueras et al., 2000).

In two breast fed children, faecal excretion of PCDD/Fs was found not to decrease during the weaning period to the same extent as the estimated decrease in exposure/uptake resulting from substituting breast milk with a solid diet (Abraham et al., 1996), implying that the faecal concentration in these two infants was at least partially determined by the blood concentration.

Maternal breast milk is considered the most accessible matrix that provides reliable information related to the intake of lipophilic pollutants in the corresponding infant (Verner et al., 2013) for those chemicals for which uptake via food is the primary exposure pathway for the infant (although dust is acknowledged as another important source for some POPs) (Toms et al., 2009; Lee et al., 2014). In addition, after a decline during the first month of lactation, concentrations of lipophilic pollutants in breast milk have been found to remain steady throughout the breastfeeding period, and to be close to the initial infant blood concentrations (Abraham et al., 1994; Lee et al., 2013; Verner et al., 2013; Vigh et al., 2013).

The key aims of this study were: to assess the concentration of a range of POPs (PCBs, polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides (OCPs)) in matched infant faeces and breast milk samples with reproducible results; to evaluate whether concentrations in the two media are correlated; and to evaluate whether faecal concentrations can potentially be used as biomarkers of the blood concentration of POPs in infants.

2. Materials and methods

2.1 Chemicals

Standard solutions of native PCBs, PBDEs and OCPs were purchased from AccuStandard Inc.. Isotopic carbon-labelled PCB standard solutions and PBDE standard solutions were purchased from Wellington Laboratories Inc., and isotopic carbon-labelled OCP standards were ordered from Cambridge Isotope Laboratories Inc.. n-Hexane (SupraSolv®), dichloromethane (DCM, SupraSolv®), granulated anhydrous sodium sulphate (Scharlau Chemie S.A.), silica (Davisil Grade 633, Sigma-Aldrich®) and Florisil® (Fluka, Sigma-Aldrich®) were used for extraction and clean-up. Silica and Florisil were activated at 140 °C for at least 12 hours before use. Sodium sulphate was baked at 400 °C for at least 12 hours before use.

2.2 Recruitment and sample collection

This project received ethics approval by The University of Queensland Ethics Committee (approval number H/308 NRCET). Ten mothers were recruited opportunistically when they were pregnant, from Sydney (7) and Brisbane (3), Australia. Breast milk samples were collected in August, 2013. A minimum of 50 ml of breast milk was collected from each mother over multiple collections over a one-month period into a clean glass jar that was provided. These mothers then collected faecal samples from their infants twice a week for two weeks, beginning 12-20 weeks after breast milk sampling. Only faecal material that had not been in contact with the inner liner of the diaper was collected (Supporting Information **Instruction for Sampling Faeces**). Questionnaires requesting demographic information about each mother /infant pair were completed by the mothers (Supporting Information **Tables S1, S2**). Breast milk and faecal samples were stored in a freezer at the participants' homes until transportation (on ice, in a cool bag) to the laboratory. In the laboratory, samples were stored at -20 °C until analysis.

2.3 Sample analysis

Lipid content in milk samples was determined gravimetrically in an aliquot based on a liquid-liquid extraction protocol. Approximately 1 g of homogenized milk sample was weighed in a 15 ml centrifuge tube. Then 2 ml of methanol, 1 ml of methyl tert-butyl ether (MTBE) and 1 ml of hexane were added consecutively. The centrifuge tube was shaken vigorously (10 min), sonicated (10 min) and centrifuged at 2000 rpm (10 min). The upper layer was transferred to a pre-weighed tube. Again 1 ml TBME and 1 ml hexane were added to the original tube which then was shaken (10 min), sonicated (20 min) and centrifuged at 4000 rpm (10 min). The upper layers were combined and blown down to dryness. The tube was put into a 103 °C oven and weighed periodically until a stable reading was obtained.

For contaminant analysis, the homogenized milk sample (~6 g) was transferred to a centrifuge tube and spiked with labeled standard solution (see **Table S3** in the Supporting Information). The milk was extracted as described above for lipid content measurement. After concentrating the extract to about 1 ml, it was applied onto the top of a glass column (30 cm × 1.0 cm i.d.) containing 1 cm of anhydrous $NaSO_4$, 14 cm of alumina, 14 cm of florisil and 1 cm of $NaSO_4$ from top to bottom. The sample was then eluted with 100 ml of a mixture of hexane and DCM (1:1 V:V). The eluate was concentrated to 0.5 ml and applied onto the top of another column (17 cm × 0.8 cm i.d.) containing 0.5 cm of $NaSO_4$ and 15 cm of silica gel from top to bottom. The second column was then eluted with 30 ml of a mixture of hexane and DCM (1:1 V:V). The eluate was concentrated to 25 µl and 25 µl isooctane containing 200 pg ^{13}C-PCB141 was added as the recovery standard right before instrumental analysis.

The homogenized, freeze-dried faecal samples (~3 g) from each infant were placed into 100 ml ASE cells with 10 g silica gel, spiked with internal standards and then extracted using hexane and DCM (1:1 V:V) on a Thermo Scientific™ Dionex™ ASE™ 350 Accelerated Solvent Extractor system. The program was: 100 °C, 1500 psi, 3 static cycles of 7 minutes and purge time of 120 seconds. The extracts were transferred to a column (1.2 cm i.d.) containing 20 cm silica, and eluted with 100 ml of a hexane/DCM mixture (1:1 V:V). The extract was concentrated and transferred to another column (1.2 cm i.d.) containing 20 cm florisil, and eluted with 80 ml hexane/DCM (1:1 V:V). The hexane and DCM (1:1 V:V) extractable lipid content of the samples was determined in a separate extraction employing ASE with the same conditions as above, followed by evaporation to dryness and gravimetric determination of the lipid content.

2.4 Instrumentation, Analysis and Quantification

All samples were analysed for indicator PCBs (PCB 28, 52, 101, 118, 138, 153 and 180), PBDEs (PBDE 47, 99, 100, 153 and 154) and OCPs (pentachlorobenzene (PeCB), hexachlorobenzene (HCB), heptachlor, heptachlor epoxide B, heptachlor epoxide A, mirex and p,p'-DDE) using a Thermo Fisher GC 1310-HRMS DFS operated in EI-MID mode. A DB-5MS column (30 m × 0.25 mm × 0.25 µm, J&W Scientific) was used. All the samples were analysed at a resolution ≥5000. The internal standard quantification method was used. The signal/ noise (S/N) ratio was above 10 for all quantified peaks.

2.5 Quality control

For every 5 samples analysed, a blank sample was included to check laboratory contamination. Cow's milk was used as the blank for breast milk, while diatomaceous earth was used as the blank for faeces. The method limit of quantification (mLOQ) of one compound was defined as the mean value of its concentration in blank samples plus 10 times its standard deviation, while the limit of detection was set at the mean plus 3 times the standard deviation. The data were blank-corrected by subtracting the mean concentrations of target compounds observed in the blanks. Diaper liners were also analysed as quality control samples, and low concentrations of target compounds were observed (Supporting Information **Table S4**). To minimize contamination, liners with the faecal samples were taken out of the diaper right after the infants' bowel movement and the faecal samples were then tipped into a pre-cleaned aluminium foil sheet (faecal material that had been in contact with the inner liner of the diaper was carefully retained), folded up, and put into the freezer immediately.

The average recoveries of the ^{13}C-labelled standards ranged from 30-110% (in breast milk samples) and 32-112% (in faeces samples) (Supporting Information **Table S3**). Replication of lipid analysis showed good reproducibility with a coefficient of variation (CV) of 4.5% for breast milk and 6.9% for faeces. Two replicates (i.e. subsamples of a common sample) were analyzed for three of the breast milk and all 10 faeces samples. Average coefficients of variation (CV) of the concentrations were 13% and 19% for breast milk and faeces, respectively; larger differences (20% - 40%) were observed for chemicals that were measured at relatively low concentrations (Supporting Information **Table S5, S6**). Where replicates were analyzed the mean concentration of the two replicates was used for further statistical analysis of association.

2.6 Statistical analysis

Multiple linear regression analyses examining potential associations between $\log_{10}$-transformed faecal concentrations and factors including $\log_{10}$-transformed milk concentration (data $\log_{10}$-transformed for normal distributions), other feeding characteristics, and age were performed using STATA IC 12 (College Station, TX, USA). For regression analyses, measures below limit of detection (LOD) were imputed as LOD/2; measures between LOD and the mLOQ were imputed using the average of two determinations.

3. Results and discussion

3.1 Concentration of POPs in breast milk and infant faeces

A range of PCB congeners, BDE congeners and OCPs were detected in both faeces and breast milk samples (**Table 1**; detailed information for individual samples is presented in the Supporting Information **Table S5, S6**). PCB 52, 101, PBDE 154, heptachlor and heptachlor epoxide A were detected above the mLOQ in less than half of the breast milk samples, and PBDE 154 and heptachlor epoxide A were detected above the mLOQ in less than half of the faeces samples, as indicated by the shaded rows in **Table 1**. For this reason, these 5 compounds are not discussed further. This is the first time that concentrations of these three groups of POPs in faeces have been reported in one study.

The highest concentration in breast milk was measured for PCB 153 (1.3 - 24 ng/g lipid) among PCBs, BDE 47 (0.27 - 8.5 ng/g lipid) among PBDEs and *p,p'*-DDE (9.0 - 230 ng/g lipid) among OCPs. Concentrations of individual chemicals varied typically between 1 and 2 orders of magnitude among all mothers (**Table S5).** This range is much greater than the uncertainty in the measurement of the concentrations (as indicated by the replicate analyses, see above), indicating that the dataset provides a good basis for assessing possible relationships between concentrations in breast milk and concentrations in faeces. One potential reason for this relatively large variation in concentrations in breast milk is that the study included mothers with a range of different ethnicities/origins including mothers who had lived abroad for most of their lives. Chemical specific differences in exposure between different countries/continents have been demonstrated in many studies (Solomon and Weiss, 2002). Other reasons may include differences in age and diet (Supporting Information **Table S1**).

This variation was reflected in the concentrations in faeces (**Table S6)**, which ranged from 0.44 - 12 ng/g lipid for PCB 153, 0.34 - 15 ng/g lipid for BDE 47, and 4.0 - 41 ng/g lipid for *p,p'*-DDE. For PCB 153 the lowest concentrations in both breast milk and faeces were observed in Pair 4, while the highest concentrations in both matrices were observed in Pair 6. For BDE 47, the lowest concentrations in both matrices were from Pair 7 and the highest concentrations in both matrices were from Pairs 10 and 6. The highest levels of DDE occurred in Pairs 6 and 9 for both matrices. The relationship between concentration of POPs in breast milk and in infant faeces is explored in more detail below.

3.2 Relationship between the log-transformed faecal concentration of POPs and a variety of factors

In this study, we examined the relationship between the log-transformed faecal concentration of all 14 compounds from **Table 1** and a variety of factors including: log- transformed milk concentrations; infant age in months; adjusted months of breastfeeding; fraction of total feeding due to breastfeeding (adjusted breastfeeding months/age); adjusted months of solid food; total weight gain; and rate of weight gain (**Table S2, S7**). A variety of possible interrelationships among these variables were also examined in order to identify potential co-variation that could affect the regressions.

Measured faecal concentrations for all analytes together are correlated with milk concentrations (**Fig. 1**; 14 analytes, 10 paired measurements for each; Pearson corr. Coeff. =0.72).

Adding other factors related to milk concentration in general did not result in significant independent contribution to the observed relationship. There was no significant relationship with months of breastfeeding, fraction of life breastfed, or conversely, months of solid food intake. However, because of the small number of pairs, no definite conclusions can be drawn regarding these factors based on this dataset.

Age was an independent, inverse predictor of faecal concentration ($p=0.017$) when added into the model with milk concentrations, but the relationship was very slight, and adding age resulted in only a small improvement in the adjusted R-squared (0.54 vs. 0.52 for milk concentration alone) (**Table 2**).

Similarly, absolute change in weight (but not rate of weight change) was also borderline significant when added to milk concentrations in the model ($p= 0.092$), with the findings again in the inverse direction – greater weight gain results in slightly lower faecal concentrations (R^2 of 0.53). However, those two parameters, age and change in weight, are highly correlated (Pearson corr. coeff. = 0.95), so it is not possible to determine which of the two contributed more to faecal concentrations. In summary, in this small dataset faecal concentrations are highly

predicted by milk concentrations, and other factors cannot be detected in this dataset, with the exception of a small effect of age or growth.

Two possible mechanisms are postulated for a monotonic correlation between human milk and infant faeces. The first mechanism is that some portion of the ingested chemical is excreted in the faeces without having been absorbed systemically, and that the concentration in the milk is a good surrogate for the infant's ingestion of the chemical. The second mechanism is that the chemical concentration in the faeces is controlled by the chemical concentration in the body, and that the concentration in the milk is a good surrogate for the concentration in the body of the infant during the time of breastfeeding. A mixture of these two mechanisms may also operate depending upon the specific chemical and the exposure matrix (food, dust, or other matrix). Further elucidation of these two mechanisms will likely require additional datasets, in particular, datasets with blood concentration data and including infants or small children that are not being currently breastfed as well as those that are.

3.3 Chemical-specific observations

This dataset can neither confirm nor refute the hypothesis that faecal concentration reflects blood concentration. However, we can use the ratio of concentrations of POPs in faeces samples (C_f, in ng/g lipid) to that in milk samples (C_m, in ng/g lipid):

$$R_{fm} = C_f / C_m$$

to explore which of the two explanations for the monotonic correlation between C_f and C_m might apply to the different compounds. The R_{fm} values for each compound in each pair of samples (only the pairs that have data above mLOQ) are presented in the Supporting Information **Table S8**. A compound specific summary of the data is presented in **Table 1**. The R_{fm} for individual samples ranged from 0.044 for DDE and PCB 180 to 4.2 for PCB 28, and the median R_{fm} ranged from 0.20 for PCB 153 and HCB to 1.1 for BDE 47.

For a given chemical, R_{fm} varied among mother/infant pairs by as little as a factor < 2.5 for BDE 47 up to a factor >32 for Heptachlor epoxide B (Fig. 2, Table S8, Fig. S1). The different variability in R_{fm} between chemicals indicated that different factors were involved for different chemicals in determining the relationship between C_f and C_m. If the hypothesis that faecal concentration reflects concentration in the infant's body (i.e., the second mechanism outlined above) is true, one would expect high variability of R_{fm}. This is because pharmacokinetic modeling has shown that the relationship between C_m and concentrations in the infant's body is strongly influenced by e.g., age and length of breastfeeding (Verner et al., 2013). On the other hand, if the faecal concentration reflects the amount of chemical ingested (the first mechanism outlined above), one would expect low variability of R_{fm} under certain conditions. This would be the case if the mother's milk was the dominant source of ingested chemical and the chemical's absorption efficiency did not vary widely between individuals. It would also be the case if another source besides mother's milk dominated the infant's ingestion, and if the same source also dominated the mother's exposure.

The variability of R_{fm} between pairs for the major BDEs, i.e. BDE47 and BDE153 is comparatively very small (Table 1, Fig 2). This would speak for the faecal concentrations reflecting the amount of these chemicals ingested. Dust is considered one of the primary vectors of the exposure of the mother and the infant for BDEs (Trudel et al., 2011) and it is reasonable to assume that the levels of these chemicals in mother's milk and in food/dust are correlated for mothers that are living in the same (dust) environment. The relatively large molar mass of BDEs could also resort in restricted absorption in the digestive tract (Kelly et al., 2004), which would favor this mechanism.

For remaining compounds, it is noteworthy the lowest R_{fm} values for PCB118, 138, 153,180, DDE and mirex all occur with Pair 1. Besides Pair 1, other two pairs, i.e. Pair 9 and Pair 2, have the biggest numbers of compounds (5 and 3, respectively) with the lowest R_{fm} values among all pairs. Those mother/infant pairs with the lowest R_{fm} values are pairs that have older children and the longest periods of solid food being introduced before faecal samples were collected (see **Table S8**). The combination of these factors would be expected to lower R_{fm}. In addition, PCBs and HCB have been proved to have very high absorption efficiency in gut both adults and infants (McLachlan, 1993; Abraham et al., 1994; Moser and McLachlan, 2002). This would speak more for the faecal concentrations reflecting the concentrations in the infant's body.

The primary purpose of this pilot study was to demonstrate that a large range of compounds could be measured in feces with reproducible results. The strong correlations between maternal milk and faecal concentrations, whether due to faeces levels directly reflecting milk levels, indirectly reflecting another common exposure pathway for mother and infant, or reflecting levels in the infant's body, suggest that faeces is a promising matrix

for biomonitoring infant exposure to POPs. Additional data, including blood concentration data paired to faecal data, are being generated to further evaluate the use of faecal concentrations as a marker for internal exposure of infants and children. In future work it is important that a broad spectrum of chemicals be studied as the faeces concentrations of different chemicals are clearly influenced by different factors. Longitudinal studies will also be important to see how the concentrations in faeces change over time in the same infant as a result of changes in concentrations in blood and changes in exposure vectors such as diet and dust ingestion.

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